REVIEW ARTICLE

Fluorescence correlation spectroscopy for the characterization of membranes: A short review

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Abstract

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Fluorescence correlation spectroscopy (FCS) is a highly sensitive fluorescence microscopy technique that can be used to probe a wide range of biophysical processes including diffusion, ligand-receptor binding and molecular aggregation on artificial and cell membranes. FCS is able to measure very small volumes at nanomolar concentrations. In this work, we review the different types of diffusion on cell membranes, describe the theory of FCS and illustrate several of its applications for the characterization of membranes and membrane associated proteins. For comparison with other techniques we discuss the differences of FCS and fluorescence recovery after photobleaching (FRAP), a widely used technique for diffusion measurements on membranes, in detail.

Key words : fluorescence correlation spectroscopy, autocorrelation, membrane diffusion, cell membrane

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Biological membranes play a vital function in cellular processes. Signal transduction, cell division, apoptosis and receptor-ligand interactions are strongly influenced by concentration, diffusion, chemical kinetics, hydrodynamic properties and the organizational structure of the lipid bilayers and the proteins embedded within. The fluid mosaic model described 30 years ago characterized the cell membrane as a two-dimensional oriented solution of integral proteins embedded in a matrix of a viscous phospholipid bilayer in thermodynamic equilibrium (Singer and Nicolson Garth L., 1972). The model proposes random two-dimensional diffusion in a fluid bilayer. However, it was observed that lateral diffusion coefficients for integral proteins in cell membranes a) are one to two orders of magnitude smaller than diffusion coefficients in model membranes (Jacobson *et al.*, 1987; Kucik *et al.*, 1999), and b) show a non-homogeneous distribution over cell surfaces (Wohland *et al.*, 2001a). Hence, new models were proposed to account for these observations (Jacobson *et al.*, 1995; Simson *et al.*, 1995 and references therein). Deviations from simple Brownian motion can be induced by small lipid domains of different viscosity (so-called rafts, see Simons and Ikonen, 1997) into which certain membrane proteins partition with a higher degree. It was shown that barriers exist that obstruct transiently the diffusion of proteins (Edidin *et al.*, 1991). Immobile fractions of membrane proteins detected by fluorescence recovery after photobleaching (FRAP, for a description of this technique see Wolf, 1989) are not just represented by stationary proteins but by interaction with proteins associated with the cytoskeleton (Zhang *et al.*, 1991). And there are proteins that undergo directed motion towards the cell edge propelled by cytoskeleton motors (Sheetz *et al.*, 1989).

For the past three decades, FRAP has been the major tool for monitoring the lateral diffusion of molecules in cell and model membranes. It can measure diffusion coefficient and the fraction of labeled membrane components that is mobile and immobile. Another method for diffusion coefficient determinations is single-particle tracking (SPT) which traces the motion of a single molecule on the cell surface over a period of time. The individual particle is labeled with a fluorophore or a gold colloid and observed with high resolution digital imaging microscopy (Saxton, 1997). Analysis of SPT measurements can determine the protein dynamics in the membrane including random diffusion, anomalous diffusion, directed motion and immobility (Saxton and Jacobson, 1997).

The purpose of this article is to describe a method that has recently been gaining interest in the analysis of biomolecules in solutions and on cell surfaces. Fluorescence correlation spectroscopy (FCS) is a versatile technique used to measure diffusion coefficient of molecules and particles. FCS is the statistical analysis of time-dependent fluorescence intensity fluctuations in a confocal observation volume. The autocorrelation functions (ACFs) can be derived from the temporal fluctuations and can be predicted from the type of underlying process, for instance translational diffusion or chemical reactions. Magde *et al.* (1972) introduced FCS for the first time in the 1970s and it is now an important tool to probe biomolecular processes *in vitro* (Rauer *et al.*, 1996; Van Craenenbroeck and Engelborghs, 1999; Wohland *et al.*, 1999) and in vivo (Brock *et al.*, 1998; Schwille *et al.*, 1999a; Wachsmuth *et al.*, 2000; Cluzel *et al.*, 2000). The capability of FCS to measure rotational and translational diffusion in membranes has been exploited by several authors (e.g. Elson, 1986; Rigler *et al.*, 1999).

In the present work we describe the basics of FCS and its application to membrane measurements and will discuss the advantages and disadvantages of this technique compared to FRAP.

Diffusion

The translational and rotational diffusion of a molecule in biological membrane is described as a cylinder with radius a and height h moving along the xy-direction and rotating along the z-axis. (Saffman and Delbrück, 1975). In an isotropic environment, diffusion of a molecule is defined by Brownian motion

$$\langle x^2 \rangle = 4D_t t \qquad \langle \theta^2 \rangle = 2D_r t \qquad (1)$$

where $<x^2>$ and $<\theta^2>$ are the mean square displacement or angular rotation in time t. D_t and D_r are the coefficients of translational or rotational diffusion, respectively.

In biological membranes, if the viscosity of the fluid surrounding the membrane is taken into account, the translational and rotational diffusion coefficients are related to the mobility of the molecule (Saffman and Delbrück, 1975)

$$D_t = \frac{kT}{4\pi\mu h}\left(\ln\frac{\mu h}{\mu' a} - 0.5\right) \qquad D_r = \frac{kT}{4\pi\mu a^2 h} \qquad (2)$$

where k is Boltzmann's constant, T is the absolute temperature; μ is the viscosity of the membrane and μ' the viscosity of the exterior fluid.

Anomalous diffusion

The eq. 1 and 2 apply for ideal cases where Brownian motion is dominant. But in biological membranes it is often observed that the mean square displacement is not linearly proportional to time, as is the case for Brownian motion. This phenomenon is called anomalous diffusion (Schwille *et al.*, 1999b and references therein). Deviations from Brownian motion can be due to restrictions in mobility of molecules caused by non-specific interaction between particles or obstruction of mobility within or by domains of lipids or proteins. In this model the mean square displacement is given by

$$\langle \Delta x^2 \rangle = \Gamma t^{\alpha} \qquad (3)$$

where the temporal exponent α ranges between 0 and 1, and the transport coefficient Γ is analogous to the diffusion coefficient but with dimensions depending on α.

FCS theory

In an FCS experiment the fluorescence intensity is measured from an open probe volume in a sample which contains fluorescent particles of interest. The probe volume is usually given by a confocal arrangement which is defined by the focal volume of focused laser beam and a pinhole that spatially filters the emitted fluorescence light to ensure that only light from the focus is detected. A typical FCS setup for measurement of some processes on cell membranes is depicted in Figure 1. The fluorescence intensity shows characteristic fluctuations $\delta F(t)$ around the average fluorescence signal $<F>$. These fluctuations are caused by molecular processes and contain therefore information on their nature. The fluctuations might be due to processes that change the fluorescence quantum yield or absorption coefficient of the particles (e.g. intersystem crossing into a triplet state or a cis-trans conformational change that renders the fluorophore non-fluorescent as long as it resides in this state). Or they can be produced by molecular motions which induce fluctuations in the number of fluorescent particles (translational diffusion) or in the alignment of their excitation and emission dipoles in respect to the excitation polarization and the emission polarized detection (rotational diffusion). In order to obtain information about the underlying molecular process, these fluctuations can be analyzed in terms of a fluorescence intensity autocorrelation function (ACF) that is calculated by (Elson and Magde, 1974; Thompson, 1991).

$$G(\tau) = \frac{\langle F(t)F(t+\tau)\rangle}{\langle F(t)\rangle^2} = \frac{\langle F(0)F(\tau)\rangle}{\langle F(0)\rangle^2}$$
$$= \frac{\langle (\langle F\rangle + \delta F(0))(\langle F\rangle + \delta F(\tau))\rangle}{\langle F(0)\rangle^2}$$
$$= \frac{\langle \delta F(0)\delta F(\tau)\rangle}{\langle F(0)\rangle^2} + 1 \qquad (4)$$

The angular brackets <> indicate a time average, F is the fluorescence signal as a function of time, and τ is the correlation time. δF denotes fluorescence fluctuations around the mean value. The transition form the first part of the right hand side in eq. 4 to the second part is possible

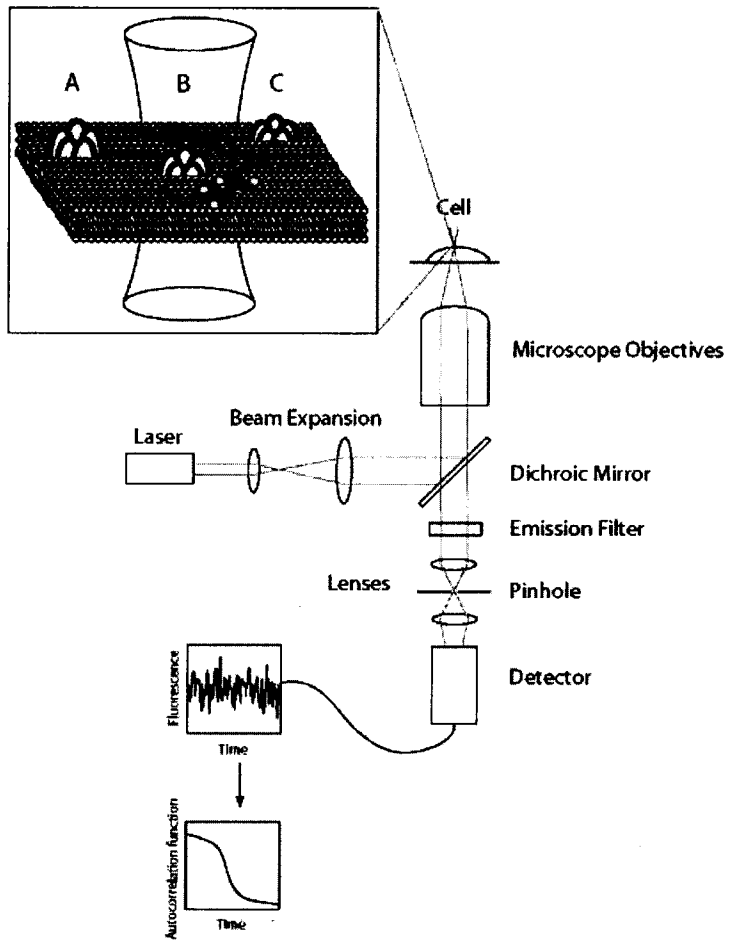

Figure 1. A typical FCS setup. An argon/krypton ion laser is used for the excitation of the fluorophores. The laser beam is expanded with two achromat or biconvex lenses and reflected with a dichroic mirror and is then focused with a microscope objective onto the cell surface. The fluorescence emission is then collected by the same objective and is separated from the excitation beam by the dichroic mirror. The fluorescence passes an emission filter, is spatially filtered by a pinhole and focused onto a detector (avalanche photodiode or photomultiplier). It is important to have a small sample volume which is determined by the pinhole diameter and the laser focus dimensions. The fluorescence signal measured by the detector is autocorrelated. Various parameters depending on the biophysical processes can be derived from the autocorrelation functions. In experiments to measure processes faster than the dead-time of the detector, two detectors can be used and the signals cross-correlated. In such a setup, a beam splitter is placed after the pinhole to divide the emission before collection by the detectors. Cross-correlation can also be applied to the investigation of two fluorophores or labeled molecules emitting at different wavelengths. In this case, instead of a beam splitter, another dichroic mirror is placed after the pinhole to separate the different emission wavelengths. Emission filters in front of the detector is necessary to filter out the excitation and background light. Some of the processes that FCS can measure for two-dimensional diffusion on a cell membrane are depicted in the figure above. (A) Directed motion or flow along the y-direction through the probe volume, (B) free brownian motion in and out of the probe volume causing fluctuations in fluorescence intensity and (C) anomalous diffusion caused by obstructions or interactions with domains of proteins or lipids in the membrane.

because we assume that the observed processes are stationary, i.e. their statistical properties are invariant to a shift in time. Eq. 4 shows how the ACF can be calculated directly from the fluorescence intensity signal and how this is related to the autocorrelation of the intensity fluctuations.

The fluorescence intensity from a small illuminated probe volume can be written as:

$$F(t) = Q\int I(\vec{r})S(\vec{r})CEF(\vec{r})C(\vec{r},t)d\vec{r} \qquad (5)$$

Here Q-the fluorescence yield of a particle - is the product of the absorption coefficient and the quantum yield of the fluorophore, and the detection efficiency of the instrument including the detector; r is a spatial coordinate, $I(r)$ is the intensity profile of the excitation light; $S(r)$ describes the extension of the sample; $CEF(r)$ is the collection efficiency function that characterizes the effect of the pinhole on the detected light intensity (for description of pinhole/CEF see (Qian and Elson, 1991; Rigler *et al.*, 1993; Wohland *et al.*, 2001b); $C(r,t)$ is the function that describes the positions of the particles at time t. With the Gaussian intensity profile

$$I(r,t) = \frac{2P}{\pi w_0^2} e^{-2r^2/w_0^2} e^{-z^2/z_0^2} \qquad (6)$$

and assuming that the particles are non-interacting and the positions of the particles are governed by simple Brownian motion one can show (Elson and Magde, 1974) that the solution to eq. 4 is

$$G(\tau) = \frac{\gamma}{N} g_i(\tau) f_i(\tau) + G_\infty \qquad (7)$$

where N is the number of particles in the probe volume, the correction factor γ is necessary to account for the non-homogeneous intensity distribution in the focal plane and is $^1/_2$ in the 2D case, and $1/\sqrt{8}$ in the 3D case. $g_i(\tau)$ is a function that describes translational diffusion, $f_i(\tau)$ is a function that describes other processes that cause fluctuations in addition to the translational diffusion, and G_∞ is the convergence value of the ACF for long times (usually 1, but it is often advantageous to keep it as a fit parameter). Table 1 shows ACFs for different types of diffusion and non-diffusion processes.

If several components with different quantum yields are present then (Thompson, 1991)

$$G(\tau) = \frac{\sum_{j=1}^{R} \alpha_j^2 Y_j N g_{ij}(\tau) f_{ij}(\tau)}{\left[\sum_{j=1}^{R} \alpha_j Y_j N\right]^2} \qquad (8)$$

Here $\alpha_j = Q_j/Q_1$, where the fluorescence yield Q_j is a product of absorbance, fluorescence quantum efficiency and experimental fluorescence collection efficiency of the jth component. Y_j is the mole fraction for each species j with N the average number of particles in the probe volume. Figure 2 shows simulated ACFs for two-dimensional diffusion showing the dependence on different parameters from Table 1.

Applications of FCS in membranes

There have been numerous applications of FCS *in vitro* on the measurement of fluorescent labeled biomolecules in solution. One of the first groups to study lateral diffusion on cell membranes *in vivo* was Elson *et al.* They measured the diffusion coefficient of a labeled lectin concanavalin A binding to rat myoblast plasma membrane receptors and compared it with the prevalent FRAP method (Elson *et al.*, 1976). Thompson's group (Palmer and Thompson, 1989) developed the theoretical and experimental study of submicroscopic clusters of fluorescent molecules. This can be applied to the investigation of aggregation of protein receptors which is important for signal transduction pathways in cells. Problems with photobleaching and the limitation of FCS in measuring slower diffusing molecules led Petersen's group to develop a new approach. Scanning fluorescence correlation spectroscopy (S-FCS) to study dynamic processes of aggregates in cell mem-

Table 1. Functions for various diffusions and non-diffusion processes that can be substituted into equation (4) to determine the autocorrelation function

Types of diffusion	γ	$g_i(\tau)$	References
2-D translational	$\frac{1}{2}$	$\frac{1}{N}\left(\frac{1}{1+4D\tau/\omega_1^2}\right)$	(Elson and Magde, 1974)
3-D translational	$\frac{1}{\sqrt{8}}$	$\frac{1}{N}\left(\frac{1}{1+4D\tau/\omega_1^2}\right)\left(\frac{1}{1+4D\tau/\omega_2^2}\right)^{1/2}$	(Aragon and Pecora, 1976)
Anomalous	$\frac{1}{2}$	$\frac{1}{N}\left(\frac{1}{1+\Gamma\tau^{\alpha}/\omega_1^2}\right)$	(Schwille *et al.*, 1999b)
Flow	$\frac{1}{\sqrt{2}}$	$\frac{1}{N}(e^{-(\tau V/\omega_1)})$	(Magde *et al.*, 1978;Thompson, 1991)
Other process		$f_i(\tau)$	
Rotational diffusion in membranes		$R(1-e^{-\tau/\tau_r})$ where $\tau_r = \frac{1}{2D_r}$ $D_r = \frac{kT}{4\pi\mu a^2 h}$	(Aragon and Pecora, 1976; Kask *et al.*, 1989; Saffman and Delbrück, 1975)
Triplet-state		$(1 - T_{trip} + T_{trip}e^{-\lambda_{trip}\tau})$	(Widengren *et al.*, 1994)
Trans/cis isomerization		iso $\underset{k_{biso}}{\overset{k_{iso}}{\rightleftharpoons}}$ [singlet $\rightleftharpoons$ triplet] $1 + \frac{K_{iso}}{1-K_{iso}}e^{-k_1\tau}$ $K_{iso} = \frac{k_{iso}}{k_{iso} - k_{biso}}$ $k_1 = k_{iso} + k_{biso}$	(Widengren *et al.*, 1999)

ω_1 is the laser beam waist
ω_2 is the axial distance in the confocal volume where fluorescence intensity has decreased by $1/e^2$
Γ is the transport coefficient where the mean square displacement becomes $\langle\Delta x^2\rangle = \Gamma\tau^{\alpha}$ and α is the temporal exponent $0 < \alpha < 1$. Γ is analogous to the diffusion coefficient
V is the constant speed of the non-translating particles in y-direction through the sample volume
R describes coefficients for rotational correlations which depend on the geometry of the molecule and the polarization sensitivity of the detection
T_{trip} is the population in the triplet state at equilibrium
λ_{trip} is related to the rate of population accumulation in the triplet state
k_{iso} is the rate of formation of the singlet or triplet state from the isomer
k_{biso} is the rate of regeneration of the isomer from the singlet or triplet state. This is based on the assumption that photo-induced isomerization occurs from the singlet and triplet state equally

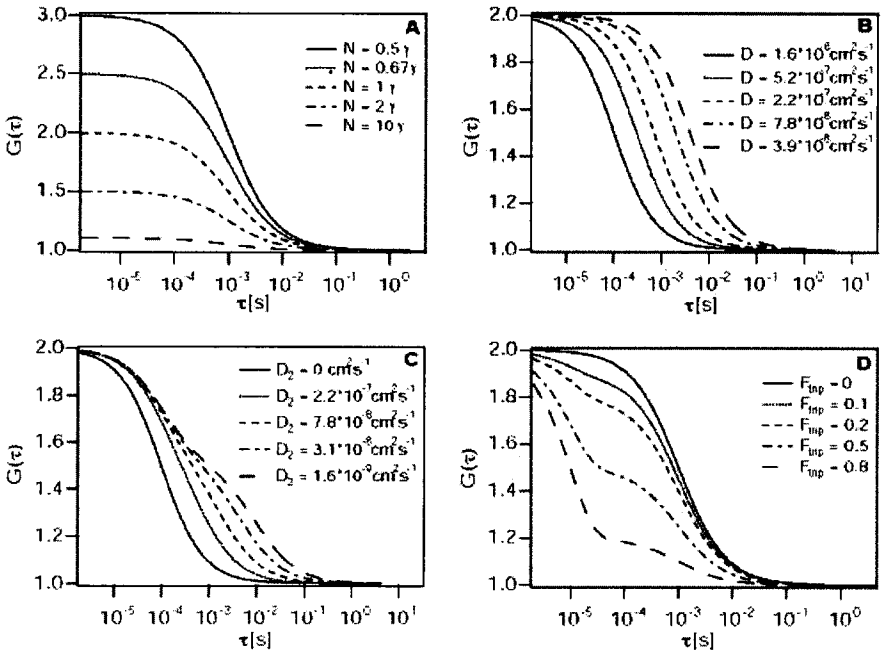

Fig. 2
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Figure 2. Calculated ACFs for the 2D case showing the dependence on different parameters.

A) Concentration dependence of the ACF. The values of N are given in respect to γ, the correction coefficient, which is $^1/_4$ in the 2D case (see Eq. 7). $D_1 = 1.6\ 10^{-7}\ cm^2s^{-1}$.

B) Dependence of the ACF on the diffusion coefficient D.

C) Typical ACFs when two different particles are present. The first particle species is assumed to have a diffusion coefficient of $D_1 = 1.6\ 10^{-6}\ cm^2s^{-1}$. The second diffusion coefficient D_2 is given in the figure. All particles are supposed to have equal molar fractions.

D) Particles are supposed to possess a triplet state. As shown, the ACF depends strongly on the fraction of molecules that reside in the triplet state (F_{trip}) at anytime.

branes (Petersen, 1984). In S-FCS, different regions of the cell membrane are probed as the cell is translated at a known velocity slower than the diffusion of the fluorescing molecules under the laser beam. S-FCS was applied to the investigation of distribution of antibodies specific to EGF receptor where the average cluster density and size of the EGF receptor were determined (St-Pierre and Petersen, 1992). An extension of this method for use with a confocal laser scanning microscope is Imaging correlation spectroscopy (ICS) (Petersen *et al.*, 1993). Image cross-correlation spectroscopy (ICCS) which cross-correlates the images taken at different times was used to measure the diffusion of transferrin receptor clusters in the membrane of 3T3 fibroblasts and HEp2 carcinoma cells (Srivastava and Petersen, 1998). Diffusion coefficients of aggregates measured were in the order of 10^{-12} cm^2s^{-1} which is considered immobile in FRAP. Thompson's group demonstrated the feasibility of using a charge-coupled device (CCD) detector to measure pixel-to-pixel fluorescence intensity fluctuations and spatially autocorrelating them. Named Imaging-FCS (I-FCS), it can measure the average cluster density and size but has limitations on studying dynamic biophysical

processes and chemical kinetics in cell membranes.

Recently, Schwille *et al.* (Schwille *et al.*, 1999b) reported the application of FCS to detect diffusion in lipid bilayers with single-molecule sensitivity and low enough laser power to prevent photobleaching. The diffusion of labeled lipids was proposed to be either due to at least two diffusing components or anomalous subdiffusion. A study on proteins was carried out on dye molecules and IgE receptors in cell membranes with two- and one-photon excitation (2PE and 1PE) with optimized pinhole sizes (Schwille *et al.*, 1999a).

The study of ligand-receptor binding on cell membranes by FCS is of pharmaceutical significance and this has been applied to high-throughput drug screening (Sterrer and Henco, 1997). Rigler *et al.* demonstrated the therapeutic importance of proinsulin C-peptide in diabetes using FCS to measure the equilibrium association constant to renal tubular cells (Rigler *et al.*, 1999). The structural requirements of the C-terminal for C-peptide binding were determined by analysis of the shifts of ACF curves on the displacement of receptor-bound labeled C-peptide with different ligands (Pramanik *et al.*, 2001a). With increased spatial resolution and sufficient sensitivity to analyze single molecules, FCS has been used to detect heterogeneity in diffusion times for GAL receptor in insulinoma cells (Pramanik *et al.*, 2001b) and EGF receptors (Pramanik and Rigler, 2001) suggesting different subpopulations or subtypes of membrane receptors undetected before.

The 5-hydroxytryptamine receptor type 3 ($5HT_{3A}$-R) has been characterized by Wohland *et al.* (Wohland *et al.*, 2001a) *in vitro* and *in vivo*. The homopentameric $5HT_{3A}$-R is of medical interest because several of its ligands have therapeutic effects on depression, anxiety and migraine. Equilibrium constant and molecular mass of the ligand-$5HT_{3A}$-R complex were determined by FCS. Ligand-receptor stoichiometry was deduced to be 1:1 as the number of fluorescent particles remained constant with increasing concentration of $5HT_{3A}$-R. FCS allows measurements in solution and on cell membrane to be carried out within one experiment. The fastest correlation time (< 0.3 ms) with diffusion coefficient ($D_1 \sim 10^{-7}$ to 10^{-6} cm^2/s) corresponded to the free ligands in solution. The second correlation time (1-10 ms) with diffusion coefficient ($D_2 \sim 10^{-9}$ to 10^{-8} cm^2/s) was due to non-specific binding of the ligands to lipids or other membrane molecules. The third correlation time (20-400 ms) with diffusion coefficient ($D_3 \sim 10^{-10}$ to 10^{-9} cm^2/s) was attributed to receptor-bound ligands. Measurements on HEK293 cell membranes showed three time regimes with different diffusion coefficients as shown in Table 2. The large variations of D measured in different cells and regions indicated that the diffusion of $5HT_{3A}$-R was not homogeneous throughout the cell membrane. Figure 2 shows the intensity trace and ACFs of the $5HT_{3A}$ receptor measured on a HEK 293 cell.

Comparison between FCS and FRAP

Fluorescence Recovery after Photobleaching (FRAP) also known as Fluorescence Photobleaching Recovery (FPR) is a widely used method to analyze lateral diffusion in membranes (Axelrod D, 1976). Several theoretical and experimental comparisons have been made between FRAP and FCS (Elson *et al.*, 1976; Elson, 1985; Meseth *et al.*, 1999). In FRAP, a small area of the cell membrane is irreversibly photobleached with an intense laser beam in a short time. The recovery time of the fluorescence is measured as the fluorophore labels diffuse back into the bleached region. Measured parameters include the recovery time and the level of recovery. From the recovery time the diffusion coefficient can be deduced, and from the level of recovery one can estimate which fraction of the molecules is mobile and which immobile. FRAP can be used to measure particles with D between 10^{-6} and 10^{-11} cm^2/s (Koppel *et al.*, 1976). Particles with $D < 10^{-12}$ cm^2/s are considered to be immobile.

In FCS on the other hand, it is not necessary to bring the system to an initial non-equilibrium state but all experiments rely on the fluctuations around equilibrium. Hence, FCS eliminates possible effects of photobleaching on cell sur-

Table 2. Selected results of some diffusion coefficients of molecules on cell membranes measured with FCS

Molecules	Cells	τ / ms	D/cm^{2}s^{-1}	References
transferrin receptor clusters	Mouse 3T3 fibroblasts Human HEp2 carcinoma		$1.9\pm1.0 \times 10^{-12}$ $1.5\pm1.2 \times 10^{-12}$	(Srivastava and Petersen, 1998)
EGF receptor	Rat bladder carcinoma	15	7×10^{-9}	(Widengren and Rigler, 1998)
diI-C_{12} dye	Rat basophilic leukemia	3±2 100±60	2.2×10^{-7} - 4×10^{-9} 1 - 5.6×10^{-9} $\Gamma = 8\pm0.6 \times 10^{-9}$ cm^2/s$^{\alpha}$ $\alpha = 0.74\pm0.08$	(Schwille *et al.*, 1999b)
ODR dye	Rat basophilic leukemia		$0.5\pm0.3 \times 10^{-8}$ (1PE, 100μm) $0.7\pm0.4 \times 10^{-8}$ (1PE, 25μm) $0.9\pm0.4 \times 10^{-8}$ (2PE) $0.04\pm0.03 \times 10^{-8}$ (1PE, 100μm) $\Gamma = 0.08\pm0.05 \times 10^{-8}$ cm^2/s$^{\alpha}$ $\alpha = 0.8\pm0.1$	(Schwille *et al.*, 1999a)
C-peptide receptor	Human renal tubular	80 1	1.95×10^{-9} 1.56×10^{-7}	(Rigler *et al.*, 1999)
GAL receptor	Insulinoma	22 700	7.1×10^{-9} 2.2×10^{-10}	(Pramanik *et al.*, 2001b)
EGF receptor	Human diploid fibroblasts	3 100	5.2×10^{-8} 1.6×10^{-9}	(Pramanik and Rigler, 2001)
mGR receptor	Mouse pituitary	200	3×10^{-10}	(Maier *et al.*, 2002)
$5HT_{3A}$ receptor	HEK293	< 100 100 - 200 > 200	$5.5\pm1.0 \times 10^{-9}$ $2.6\pm0.5 \times 10^{-9}$ $0.9\pm0.5 \times 10^{-9}$	(Wohland *et al.*, 2001)

faces. Furthermore, FCS can measure down to the nanosecond range and thus can easily measure fast diffusion and other fast non-diffusive processes that are not accessible by FRAP (e.g. diffusion in solution, triplet states). However, slow diffusion coefficients are more difficult to measure by FCS, and the cutoff lies on the order of 10^{-9} cm^2/s, i.e. when molecules need seconds to diffuse through the focal volume of 0.25 μm diameter. The practical limit for FCS is here given by cell movements, and by the vibrational damping characteristics of the setup.

Another difference between the two methods is the concentration range accessible by the two methods. A FRAP experiment requires at least 100 fluorophores/μm^2 (Wolf, 1989). FCS experiments on the other hand, work even when less than 1 molecule is in the probe volume on average. While the signal-to-noise ratio rises in FRAP with higher concentrations, it is the opposite for

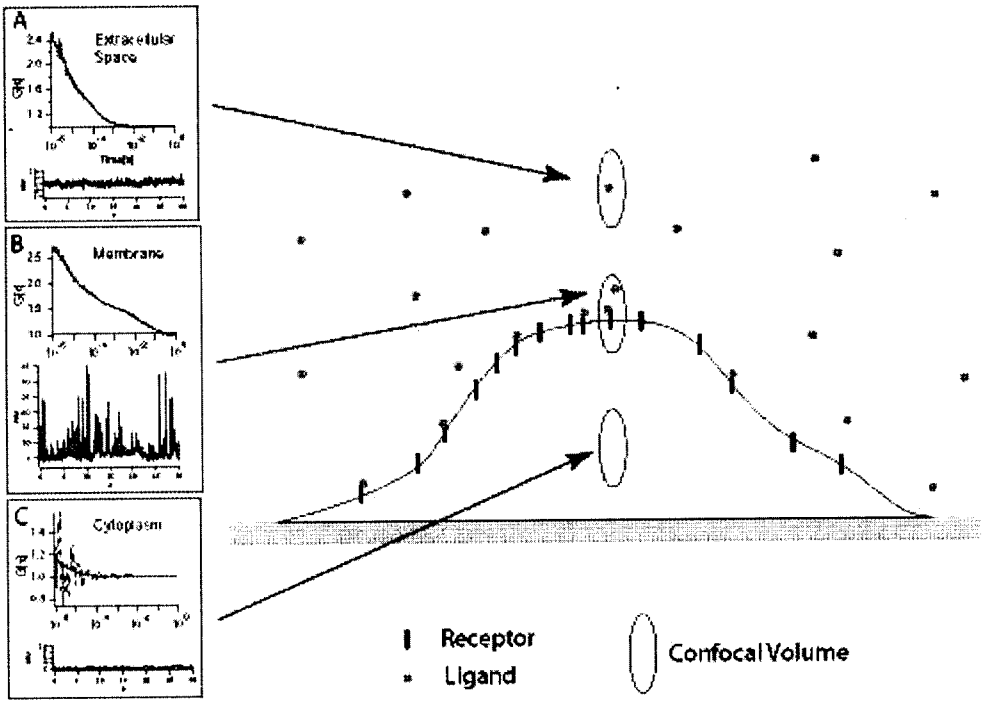

Fig. 3
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Figure 3. FCS measurements on a HEK 293 cell that expresses the $5HT_{3A}$ receptor. The ligand GR119566X (Glaxo Wellcome, Geneva Switzerland) labeled with a Cy5 fluorophore (Amersham Bioscience) was added to the cell medium (PBS buffer, pH=7.4). Measurements were taken at different positions around the upper cell membrane. In each measurement A-C the intensity trace is shown (bottom graph) and the ACF (upper graph).

A) Measurement in buffer outside the cell. Only freely diffusing ligand can be seen (D~10^{-6} cm^2/s), average count rates are around 6 kHz.

B) Measurement on the membrane. It can be clearly seen from the intensity trace that single slowly diffusing particles are detected on the membrane. We interpret this as receptors or possibly receptor aggregates that have fluorescent ligands bound and diffuse in the membrane. From the ACF we can detect at least three diffusion coefficients: D_1~10^{-6} cm^2/s for particles diffusing free in solution, D_2~10^{-8} cm^2/s for non-specifically bound ligands to the lipid membrane, and a slower D_3~10^{-9} cm^2/s for receptor bound ligands.

C) Measurements in the cytoplasm. Count rates are very low, around 2 kHz (corresponding to autofluorescence from the cell) and no correlation can be seen, indicating that no ligand was able to penetrate into the interior of the cell, as would be expected. All measurements were done with a HeNe laser at 633 nm and a power of 0.7 mW to avoid photobleaching.

FCS. Since the ratio between the amplitude of intensity fluctuations and the average intensity value decreases with increasing number of molecules the signal-to-noise ratio is actually better for FCS at low concentrations. The accessible concentration range for FCS is between 0.05 and

10000 fluorophores/μm^2.

Accurate determination of the diffusion coefficient in FRAP at low signal level is hampered by noise, making two diffusing components difficult to resolve. Gordon *et al.* (Gordon *et al.*, 1995) performed simulations to calculate the success of extracting D and differentiating between one and two-component systems under different parameters: sampling time, beam diameter and signal level. It was found that at 316 counts/channel (channel width 20ms) D should differ by a factor of 10 to distinguish between one and two components. Beyond that, reliability decreases. Time resolution of FCS investigated theoretically and experimentally on Rhodamine 6G and Rhodamine-labeled bovine serum albumin (Meseth *et al.*, 1999) determined that to distinguish two components, D must differ by a factor of at least 1.6.

Photobleaching, on the other hand, is assumed to be negligible in FCS. Therefore, it is important to consider the photostability of fluorescent dyes used in FCS. Given that the laser power used in FCS which may be in the order of several 100 kW/cm^2, photobleaching can cause problems in measuring the ACF accurately. Therefore, dyes with high absorptivities, high fluorescence quantum yields and low photodegradation quantum yields are chosen for use in FCS.

Autofluorescence of biological cells can pose a major problem in ultrasensitive FCS applications especially at wavelengths around 500 nm. Intrinsic autofluorescent biomolecules include flavins, flavoproteins, collagen and elastin. An optimal level of dye concentration that discriminates against autofluorescence background and produce reliable ACF curves is required. Conversely, concentration of the dye analyzed should be higher than the detected autofluorescence for a good resolution of the ACF curve.

FCS can provide data that is inaccessible to FRAP. FRAP measures the diffusion coefficient and the ratio between mobile and immobile fractions. FCS on the other hand measures the concentration and allows the study of chemical reaction kinetics of ligand-receptor binding, aggregation and diffusion in cell membranes. In addition FCS can yield information on other processes as for instance rotational diffusion, triplet state characteristics, and chemical reactions.

Therefore FCS and FRAP are complementary methods that give access to different parameters as well as to different concentration and diffusion coefficient ranges.

Concluding remarks

FCS has proven to be a versatile technique for both *in vitro* and *in vivo* measurements. We have discussed in this work especially its application to measurements on artificial and natural membranes. We have given the theoretical background for these measurements and have compared FCS to the widely used method of FRAP. Advantages of FCS are the large dynamic range in the time domain that allows the measurement of processes with characteristic times between nanoseconds and seconds. Hence, FCS can be used to analyze proteins bound to membranes ($D \sim 10^{-8}$ - 10^{-9} cm^2/s) and in solution ($D \sim 10^{-6}$ - 10^{-7} cm^2/s) simultaneously. In addition, FCS has a large accessible concentration range of 0.05 and 10000 fluorophores/μm^2 (0.01 nM to roughly 10 μM in solution) and has a measurement volume of less than 1 femtoliter, or about 0.2 μm^2 for membranes.

The disadvantage of FCS lies in its limited resolution of diffusion coefficients. A factor 1.6 (corresponding to a change in mass of a factor 4) between diffusion coefficients of different particles is needed to allow detection. From the applications shown, FCS has found increasing use on the study of cell and model membranes. It provides single-molecule sensitivity to analyze the diffusion of membrane components, receptor-ligand interactions and other chemical reaction kinetics. Molecular dynamics such as binding constants, diffusion coefficients and stoichiometry of reaction can be determined from changes in mobility when bound to membrane proteins. FCS is thus a powerful tool for the characterization of membranes in biological cells.

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